
Transgenic Approaches to Improve Resistance to Nematodes and Weevils

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Abstract

Banana and plantain are important staple crops for Africa and important fruit crops for Asia, Latin America and Caribbean islands. Several nematode species and rhizome weevil (*Cosmopolites sordidus*) are major pests in banana that cause heavy damage and revenue loss. Pesticides and bio-control agents control the pests, but pesticide residues pose severe environmental problems. Conventional breeding is a difficult and slow process due to the limited sources of resistance, sterility of cultivated banana varieties, polyploidy levels, long cropping cycle and the lack of rapid screening methods. Genetic engineering is considered as one of the eco-friendly and safer methods to control these pests. This review discusses the seriousness of the problem, the status and source of pest resistance and the mechanisms involved. The availability of various genes with potential to control nematodes and weevils is discussed. Further, current efforts and future prospects for identifying natural resistance genes and RNAi-based defences with potential to control nematode and banana weevil in a transgenic approach are outlined and discussed. Nematode-resistant transgenic banana cultivars expressing rice or maize cystatin genes and peptides evaluated under field conditions and those weevil-resistant cultivars

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developed using papaya cystatin gene with enhanced inhibitory potential are discussed in the light of biosafety concerns.

Keywords

Nematode and weevil resistance • Protein and peptide based transgenic defences • Cystatin genes and peptides • Plant lectins • Insecticidal proteins • Alpha-amylase inhibitors and chitinase enzymes • RNAi-based defences

17.1 Introduction

Banana and plantain (*Musa* spp.) are cultivated in over 130 countries worldwide covering approximately 10 million hectares with an annual production of 139 million tons (FAOSTAT 2014). It forms an important staple diet of Latin American and Caribbean islands, grown mainly for subsistence and for local sale. In recent years, the income through banana cultivation has reduced drastically due to severe problems affecting banana cultivation. Several nematode species and rhizome weevil (*Cosmopolites sordidus*) are major pests that cause huge production losses.

Nematodes are key pests in many commercial dessert banana plantations, and they also damages both cooking banana and plantains. Root systems damaged by nematodes are less able to utilize nutrients and water, become susceptible to secondary infection and provide weakened anchorage to the plant. Plants with weakened root systems are prone to toppling, especially in strong winds and when bearing fruit leading to loss of the fruit. Banana suffers estimated losses to nematodes of 6 M tonnes/year, representing the consumption need of 60 M people in banana-dependent countries. Weevil is most severe in plantains and East African highland bananas. Weevils damage the corm of plants by making tunnels and rootstock. Damaged corm interferes with root initiation and sap flow in the plant, resulting in yellowing of leaves and wilting of plants particularly the young suckers. The suckers finally die, whereas the older plants are retarded in their growth and produce small bunches. The weevil-damaged plants can easily be blown over by the wind. Pesticides are usually

used to control nematodes and weevils, but pesticide residue is a main concern considering the environmental and health problems. Genetic transformation is considered as one of the eco-friendly approaches for controlling weevils and nematodes.

17.2 Nematodes

Nematodes cause heavy damage to banana and plantain crops with approximately a 20% reduction in productivity globally (Sasser and Freckman 1987). In areas prone to tropical storms and particularly in Africa, losses of 40% or greater can frequently occur. Nematicide application experiments in West Africa have shown that after three crop-cycles, the potential reduction in yields is $71 \pm 16\%$ (Atkinson 2003), a region of the world where bananas and plantains provide >25% of the carbohydrate intake of approximately 70 million people, corresponding to 10% of their food energy (Ortiz and Vuylsteke 1996; Robinson 1996). Several nematodes are major pests of banana and plantain crops (Atkinson 2003; Brentu et al. 2004; Gowen and Quénéhervé 1990). *Radopholus similis* is considered the most damaging species. It has a life cycle of 20–25 days which makes its population multiply rapidly and cause severe crop loss (Bridge et al. 1995; Haegeman et al. 2010; Price 2006). In severe infections with *R. similis*, yield losses due to stunted growth are compounded by increased plant toppling in strong winds as a result of reduced root system anchorage (Gowen and Quénéhervé 1990). In the absence of *R. similis*, *Pratylenchus coffeae*, *Helicotylenchus multicinc-*

tus and *Meloidogyne* spp. can also become severe constraints on banana plantations (Bridge et al. 1995; McSorley and Parrado 1983; Brentu et al. 2004; Price 2006). Secondary fungal and bacterial infections frequently compound the direct damage nematodes cause (Duncan and Moens 2006).

Nematicides are widely applied to soils in intensive banana plantations, but they are environmentally toxic and a risk to human health (Atkinson et al. 2003). The prevalence of banana and plantain cultivation on small plots across the tropics also means that nematicides are inappropriate on the basis of both cost and grower safety. Nematode-tolerant and nematode-resistant banana cultivars have been identified, though the cultivars are only effective against single species of nematode (Lorenzen et al. 2010; Pinochet 1988). Concomitant nematode species infection is common in banana and plantain growing areas. In Uganda, the largest producer of bananas and plantains in Africa (FAOSTAT 2014), *R. similis*, *H. multicinctus* and *Meloidogyne* spp. are present in all banana-growing regions (Kashaija et al. 1994). Work in Costa Rica on the dessert banana cultivar ‘Grand Naine’ identified *R. similis*, *P. coffeae*, *M. incognita* and *H. multicinctus* causing significant reductions in yield, with the multi-species infections causing greater damage than single species (Moens et al. 2006). Similar work with the plantain cultivar ‘Apantu-pa’ in Ghana found that while *P. coffeae* caused the largest losses, the greatest necrosis and toppling, co-infection with *M. javanica* and *H. multicinctus* caused an increase in damage to the plant over any single species infection (Brentu et al. 2004). Conventional breeding has struggled to introduce the single species resistance into economically important cultivars or develop hybrids with broad nematode resistance (Lorenzen et al. 2010). A transgenic approach for nematode resistance is strongly favoured for bananas and plantains, both because of the difficulty of developing an effective resistance through breeding and the availability of several proven anti-nematode genes.

17.3 Banana Weevil

Banana and plantain production in Africa is effected significantly by rhizome weevil which is a serious pest (Ostmark 1974; Gold 1998; Gold and Messiaen 2000; Swennen and Vulysteke 2001; Fogein et al. 2002). The banana plantation decline (Gold et al. 1999) called ‘yield decline syndrome’ in Africa is associated with this weevil. By the time the crop reaches the 4th cycle, the yield loss reaches 44% (Rukazambuga et al. 1998). Crop losses up to 100% are also reported in cases of severe infestation. Establishment of new plantations is difficult due to the persistence of this infection in soil (Sengooba 1986; Price 1994). The causal organism of this devastating disease is the weevil *Cosmopolites sordidus* (Germar 1824) (Coleoptera: Curculionidae). The weevil in its adult form is free living and black in colour and measures 10–15 mm. It is also associated with crop debris and is nocturnally active as it becomes desiccated easily. The adults inhabit a particular location and remain there for long period of time. Weevils generally do not fly. Infected planting materials disseminate the weevil. Banana weevils have long life span and low fecundity. Some may live up to 4 years, though generally for 1 year. Adults generally feed on dead or dying banana plants. They are found living under newly cut or rotting pseudostems. They can survive without feeding for several months with little moisture. They lay more than one egg per week on flowering plants and crop residues. In the holes made by the rostrum, the females place their white, oval eggs singly. On the leaf sheaths and rhizome surfaces also oviposition is noticed. The emerging larvae feed on the rhizome, stem and the pseudostem. The larvae pass through five to eight instars. The adults emerge within 5–7 weeks out under tropical conditions. Eggs do not develop below 12 °C (Gold and Messiaen 2000).

The volatiles released by the host plants attract the adult weevils which enter banana plants through cut rhizomes. The weevil attack effects roots completely resulting in reduced nutrient uptake which results in reduced plant vigour and

delayed flowering increasing plant's susceptibility to other pests and diseases. Yield is reduced due to rhizomes weakening resulting in toppling and plant death.

Several cultural practices such as keeping the plantation clean by trapping the weevils are followed, but are cost intensive. Crop sanitization will remove weevil refuges and breeding sites. Application of neem (20%) is found to be beneficial in reducing the population and oviposition. Chemical fertilizers are not affordable by farmers, besides developing insect resistance. Development of resistant plants has been suggested as a potential long-term solution for controlling the weevil.

There are not many biocontrol agents known which can control these weevils. *Tetramorium guineense* and *Pheidole megacephala*, the myrmicine ants, have been reported to have successfully controlled the weevil in Cuba. Antifeedants play a significant role in weevil resistance (Ortiz et al. 1995). Kiggundu (2000) observed that corm size, hardiness, resin/sap production and their suckering ability are significant parameters in resistance response of clones introduced in Africa. Large corm size provides greater resistance (Balachowsky 1963). Certain toxic compounds present in BB genomes imparted weevil resistance which was absent in AA genome. Corm extracts from weevil-resistant AB (Kisubi) and ABB (Pisang awak) genomes showed HPLC peaks which were absent in susceptible and resistant AA clones. African cultivars have been found to be highly susceptible to weevil infection and few Indian cultivars (Karumpoovan and Poozhachendu) highly resistant (Padmanaban et al. 2001). Certain resistant cultivars are reported in Cameroon (Fogain and Price 1994). Kiggundu et al. (2003a, b) reported that some wild diploid banana (Calcutta-4), three diploid banana hybrids (TMB2×6142-1, TMB2×8075-7 and TMB2×7197-2) and cultivars like Yangambi-Km5 and Cavendish which possess high level of resistance may be exploited as resistance source. Some cultivars like Tereza, Nalukira and Nsowe possess intermediary resistance. *Musa accuminata*, AA genome progenitor, was found to be more susceptible to weevils than *M.*

balbisiana, the BB progenitor (Mesquita et al. 1984).

Laboratory studies conducted by Kiggundu et al. (2006) on the modalities of resistance to banana weevil revealed that all cultivars were attractive to the weevil and females oviposited on all cultivars. The resistant cultivars showed lower survivorship compared to susceptible ones. Antibiosis mechanisms existed in insect resistance and not antixenosis. Larval development on corms of susceptible cultivars was inhibited by methanol extracts from resistant cultivars in the laboratory.

17.4 Genes Available for Nematodes and Weevils Resistance

17.4.1 Genes for Nematode Resistance

17.4.1.1 Protein- and Peptide-Based Transgenic Defences

Proteinase inhibitors (PI) of plant origin like trypsin, serine and cysteine, which inhibit nematode development and reduce fecundity of females, have been used to control nematodes (Kiggundu et al. 2003a, b; Urwin et al. 1997). Cysteine proteinase inhibitors (cystatins), which prevent proper intestinal digestion of dietary protein in nematodes, are well developed as anti-nematode proteins (Atkinson 2000; Urwin et al. 1995). Transgenic plants expressing cystatins can provide effective control of both cyst and root-knot nematodes. The cystatins have demonstrated effectiveness against a range of major nematode pests including in field trials with *Globodera* spp. on potato expressing an engineered rice grain cystatin (Urwin et al. 2001, 2003) and *R. similis* and *H. multicinctus* on plantain expressing a maize kernel cystatin (Tripathi et al. 2015). In glasshouse trials, the engineered rice cystatin has also provided $75 \pm 5\%$ resistance in lily against *P. penetrans* (Vieira et al. 2014), and the maize cystatin has provided $84 \pm 8\%$ resistance in plantain to a mixed population of nematodes *R. similis*, *H. multicinctus* and *Meloidogyne* spp. (Roderick

et al. 2012). Vain et al. (1998) demonstrated 55 % reduction in egg production by *Meloidogyne incognita* in transgenic rice plants expressing rice cystatin. Transgenic tomato expressing taro cystatin showed resistance against *Meloidogyne* spp. (Chan et al. 2010). The transgenic plant expressing dual proteinase inhibitor transgenes demonstrated enhanced resistance to nematodes (Urwin et al. 1998). On current evidence, it is likely that a cystatin-based defence should be effective against all economically important parasitic nematode pest of banana and plantain.

A potential drawback to a cystatin defence is that the nematode is not exposed to it until after invasion of the root; consequently, young banana plants may suffer stunting given a large enough initial inoculation of nematodes. Peptides that overcome this problem by disrupting localization and invasion of host roots by plant parasitic nematodes have also been developed. Nematodes utilize a range of chemical signals produced by plant roots to achieve a successful parasitic interaction (Reynolds et al. 2011). Two synthetic peptides with distinct modes of action have been identified that interfere with the nematode cholinergic nervous system by binding to either acetylcholinesterase or nicotinic acetylcholine receptors (nAChRs) to disrupt chemoreception (Winter et al. 2002). The acetylcholinesterase-inhibiting peptide reduces the number of female *Heterodera schachtii* on *Arabidopsis thaliana* by more than 80 %. In the same set of experiments, expression of this peptide in the root tips of potato plants resulted in almost 95 % resistance to *Globodera pallida* (Lilley et al. 2011a). The mode of uptake of acetylcholinesterase-inhibiting peptide is well documented for *H. schachtii* and *R. similis* (Wang et al. 2011; Roderick et al. 2012) and likely a highly conserved process across plant parasitic nematodes. The nAChR-binding peptide is taken up by the open-ended chemosensory sensilla within the anterior amphidial pouches and is then transported along chemoreceptive neurons to their cell bodies where nAChRs are located (Wang et al. 2011; Roderick et al. 2012). Chemoreception is only impaired when that transport had been completed (Wang et al. 2011). This peptide, when expressed in root

tips of potatoes with a cellular export signal peptide, achieved up to 77 % resistance against *G. pallida* in glasshouse and field trials (Green et al. 2012). Plantains expressing the nAChR-binding peptide achieved $69 \pm 6\%$ resistance to a mixed population of *R. similis*, *H. multicinctus* and *Meloidogyne* spp. in screen house challenges (Roderick et al. 2012) and $99 \pm 1\%$ resistance to a concomitant infection with *R. similis* and *H. multicinctus* in the field (Tripathi et al. 2015). Migratory plant parasitic nematode species like *R. similis*, *H. multicinctus* and *Pratylenchus* spp. that remain motile and infective during all developmental stages may be affected throughout their lifecycle, while sedentary endoparasitic nematodes, such as *Meloidogyne* spp., are vulnerable to sensory intervention during their infective stages prior to feeding cell initiation. Both the cystatin and chemoreception disrupting peptide defences are being deployed as a stacked defence in plantain (Tripathi et al. 2015) and East African Highland banana to ensure a broad and durable defence. However, there does not appear to be a cumulative level of resistance from having two defences present (Roderick et al. 2012; Tripathi et al. 2015).

Bt proteins have effects on free-living bacterial feeding nematodes (Marroquin et al. 2000). The Cry5B protein is toxic to wild-type *Caenorhabditis elegans*, whereas some mutants of *C. elegans* are resistant to it but susceptible to Cry6A toxin (Marroquin et al. 2000). Cry55Aa, Cry6Aa and Cry5Ba showed toxicity to *M. hapla* in an induced uptake study (Zhang et al. 2012), and Cry6Aa2 reduced *M. hapla* numbers when applied as a soak (Yu et al. 2015). Transgenic Cry5B expressed in tomato hairy roots reduced *M. incognita* numbers by 75 % compared to controls (Li et al. 2008). Plant parasitic nematode control using Bt Cry proteins has potential, but the evidence base for broad nematode species control or for efficacy in the field has not yet been developed (Wei et al. 2003). The lectin concanavalin A has been shown to suppress *M. incognita* multiplication, and others, such as snowdrop lectins, have biological activity against nematodes. Many lectins, however, have toxic effects on insects and mammals (Burrows and de Waele

1997). Toxicological safety of lectins is a major concern for commercial development and needs to be studied in depth. Transgenic expression of lectins have not yet shown enough promise to make it into crop field trials (Fuller et al. 2008; Atkinson et al. 2009; Lilley et al. 2011b).

Natural resistance genes can also offer a strategy for combating plant parasitic nematodes, and several R-genes against nematodes have been identified. The sugar beet gene *Hs1pro-1* confers resistance to the cyst nematode *Heterodera schachtii* (Cai et al. 1997). The tomato *Mi-1.2* gene confers resistance against *Meloidogyne* species and has been introduced into cultivated tomato, *Lycopersicon esculentum*, by an interspecies cross from the wild species *L. peruvianum* (Milligan et al. 1998). The *Gpa2* gene also conferred resistance against potato cyst nematode *Globodera pallida* (van der Vossen et al. 2000). The main drawbacks of R-genes are a lack of genes for resistance to banana nematodes and a tendency for a highly species-specific effect.

17.4.1.2 RNAi-Based Transgenic Defences

RNA interference (RNAi) results when double-stranded RNA (dsRNA) triggers the degradation of messenger RNA (mRNA) resulting in the silencing of specific target genes. It has proven a useful tool for functional analysis of nematode genes, including for plant parasitic nematodes (Rosso et al. 2009). Triggering of RNAi silencing in nematodes that feed on plants expressing dsRNA targeting nematode genes is currently being developed as a nematode control strategy (Lilley et al. 2007). Experiments with transgenic *Arabidopsis* expressing dsRNA from inverted repeat hairpin constructs have identified six *Heterodera schachtii* genes that when suppressed result in significant reductions in female numbers up to 64% (Patel et al. 2008, 2010; Sindhu et al. 2009). Suppression of *H. glycines* by 81–93% has been achieved in soybean plants expressing dsRNA targeting one of two ribosomal proteins, a spliceosomal protein or synaptobrevin (Klink et al. 2009), while a similarly high reduction in egg production was achieved by targeting mRNA

splicing factor *prp-17* or an uncharacterized gene *cpn-1* (Li et al. 2010).

Similar positive results have been seen for banana parasitic nematodes, particularly for *Meloidogyne* spp. A high level of resistance resulted from targeting the 16D10 gene expressed in the subventral gland cells and required for parasitism in *Meloidogyne incognita*. *Arabidopsis thaliana* plants expressing dsRNA targeting the 16D10 gene achieved 63–90% reduction in gall number and size with a corresponding reduction in egg production in *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* (Huang et al. 2006). Tobacco plants expressing dsRNA targeting either a splicing factor or an integrase gene of *Meloidogyne incognita* achieved a high level of resistance to that nematode (Yadav et al. 2006). Reduction in gall numbers by >90% for *M. incognita* on transgenic soybean roots has also been shown (Ibrahim et al. 2011). However, not all plants delivered dsRNA targeting *Meloidogyne* genes have resulted in a resistance phenotype. Silencing of the putative transcription factor MjTis11 of *M. javanica* did not significantly affect the nematodes (Fairbairn et al. 2007). Only partial resistance was achieved when *A. thaliana* plants targeted either a dual oxidase gene with a probable role in cuticle formation or a subunit of a signal peptidase, a protein complex required for the processing of secreted proteins targeted individually in *M. incognita*. Higher levels of resistance were achieved by crossing transgenic lines expressing these two defences (Charlton et al. 2010). However, a combinatorial RNAi targeted at *H. glycines* did not deliver that benefit (Bakhetia et al. 2008). Possibly transgenic silencing RNAs may saturate the RNA silencing complex reducing efficacy.

Radopholus similis is also susceptible to RNAi although the extent of silencing can vary by the region of the nematode gene targeted and from experiment to experiment (Haegeman et al. 2009). Reductions by 60% in infection to *Medicago truncatula* were achieved after soaking *R. similis* in dsRNA targeting a gland cell xylanase gene (Haegeman et al. 2009). Tobacco plants expressing an inverted repeat hairpin

construct targeting an *R. similis* cathepsin B cysteine proteinase transcript achieved 80% reduction in the number of nematodes recovered (Li et al. 2015a). Targeting an *R. similis* calreticulin resulted in a 75% reduction in *R. similis* numbers recovered on transgenic tomato plants (Li et al. 2015b). There has been no report of control of *Pratylenchus* spp. by RNAi on transgenic plants. Experiments that induce in vitro uptake of dsRNA have demonstrated transcript knockdown for troponin C (*pat-10*) and calponin (*unc-87*) genes, required for muscle structure and contraction, in *P. coffeae*, *P. thornei* and *P. zaeae*. Following treatment with dsRNA nematode movement was aberrant, and multiplication on carrot discs was significantly reduced (Joseph et al. 2012; Tan et al. 2013).

The susceptibility of *H. multicinctus* to RNAi has yet to be investigated and is hampered by the scarcity of genetic sequences available for the nematode. In contrast to the availability of complete genome sequences for the *P. coffeae* (Burke et al. 2015a, b) and *Meloidogyne* spp. (Abad et al. 2008; Opperman et al. 2008) and the ongoing sequencing of *R. similis* genome (Bird et al. 2015), a complete mitochondrial genome for *R. similis* is available (Jacob et al. 2009), which greatly increases the likelihood of identifying suitable targets.

17.4.2 Genes for Weevil Resistance

Studies on differentially expressed genes following weevil infestation was initiated in a joint project between UBPP (Uganda Banana Biotechnology Project) and FABI (Forestry and Agricultural Biotechnology Institute) of the University of Pretoria. Genes expressed during weevil infestation were compared in resistant and susceptible *Musa* varieties using techniques called cDNARDA (Representational Difference Analysis of cDNA) (Hubank and Schatz 1994).

17.4.2.1 Proteinase Inhibitors

Protein inhibitors are used for obtaining defence against weevils also. Expression of proteinase inhibitors naturally in plants when insect attacks

was studied during insect wounding and herbivory by Ryan (1990), Pernas et al. (2000) and Ashouri et al. (2001). As mentioned under nematodes, cysteine proteases are enzymes in the mid gut of coleopteran insects such as the banana weevil, important in the breakdown of dietary proteins. Two major proteinase classes, serine and cysteine, are present in the digestive system of insects. Lepidoptera, Dictyoptera and Hymenoptera belong to Serine proteinase, while Odoptera and Hemiptera possess cysteine proteinase activity. Cysteine proteinases are used by Coleopteran insects (Gatehouse et al. 1985; Murdock et al. 1987). A combination of both serine and cysteine proteinases is also used (Gerald et al. 1997) by pyramiding, to harvest the double advantage of both the proteins, to combat the weevil problem (Gerald et al. 1997).

The potential of phytocystatins (OC-I and papaya cystatin) in controlling the banana weevil was studied by Kiggundu et al. (2002) who analyzed, in the gut of banana weevil, protease activity. Hydrolysis of casein at an acidic pH optimum (pH) was observed in extracts from complete weevil larval guts. Alkaline pH (pH 8.0) showed lesser activity. The presence of cathepsin L and B and cysteine protease in the larval gut was evident by the hydrolysis of the specific substrates Z-Phe-Arg-MCA and Z-Arg-Arg-MCA. In addition, by using specific Bz-Arg-MCA and N-uc-Ala-Ala-Pro-Phe-MCA substrates, trypsin- and chymotrypsin-like protease activity was observed. OC-I and cystatin were produced as fusion protein with histidine tag in *E. coli* and purified. These purified proteins at 1×10^{-5} ngml⁻¹ and 2.1×10^{-5} ng ml⁻¹ showed 66.2% and 81.6% with LD50 inhibition of cysteine protease activity in the banana weevil gut homogenate. Purified OC-I at 0.6 mg cystatin g fresh weight⁻¹ inhibited larval weight gain per day when fed on banana stem disc vacuum infiltrated with the inhibitory protein. This study demonstrated that cysteine proteases are used instead of cathepsin L and B by the banana weevil for protein digestion and metabolism in the gut, while phytocystatins are potential control agents for banana weevil growth. The importance of papain-like-cysteine proteases, trans-epoxysuccinyl-L-leucylamido-

(e-guanidino) butane (E-64) on the growth and development of several coleopteran insects was reported by Fabrick et al. (2002). In GM plants, for insect control, exogenous cysteine proteinase inhibitors were used (Leple et al. 1995). The plant cystatins OsCys I and OpCys II showed extensive growth delay on *Cosmopolites sordidus* on cystatin extract media (Kiggundu et al. 2010).

17.4.2.2 Chitinase Enzymes and Alpha-Amylase Inhibitors (AI)

Chitinase enzymes and alpha-amylase inhibitors (AI) also act potentially against the weevil infestation. The chitinolytic activity on the insect cell wall protects the plants from the further damage; genetic transformation of these anti-insecticidal genes may help to develop GM banana with enhanced resistance to weevil (Morton et al. 2000). Enhanced resistance to Lepidopteran insects was seen in transgenic plants expressing enhanced chitinase activity (Ding et al. 1998). Alpha-amylase inhibitors inhibit AL-1 and AL-2, two types of amylases isolated from wild beans (*Phaseolus vulgaris*) (Le Berre-Anton et al. 1997; Morton et al. 2000). Enhanced resistance to coleopteran insects was observed in transgenic azuki beans expressing seed alpha-amylase (Ishimoto et al. 1996).

17.4.2.3 Plant Lectins and Insecticidal Proteins

Plant lectins are inhibitory to a number of organisms (Sharma et al. 2000). Lectins isolated from pea, wheat, rice and soybean are toxic to insects due to their carbohydrate binding capabilities. A lectin from snowdrop, *Galanthus nivalis* agglutinin (GNA), is toxic to several Homoptera, Coleoptera and Lepidopteran insect pests (Tinjuangjun 2002). GNA has been found to be useful in developing transgenic potato and sugarcane resistant to Peach potato aphid and sugarcane grub (*Antitrogus consanguineus*), respectively (Gatehouse et al. 1997; Nutt et al. 1999). Some of the lectins are toxic to mammals (Jouanin et al. 1998) which is a major concern (Boulter 1993), while garlic lectins are toxic only to insects, a major concern about the use of lec-

tins, and are potential candidates for weevil control (Kiggundu 2003).

For Lepidopteran control in GM crops, Bt gene technology is the most widely used (Krettiger 1997). There are more than 50 insecticidal crystal proteins among Bt genes. The proteins are solubilized in the alkaline environment of the insect's midgut when an insect feeds on Bt endotoxin protein in GM plant and become toxic to the insect causing its death. Transgenic rice developed with cry1Ab gene was found resistant to rice leaf folder (*Cnaphalocrocis medinalis*) (Ye et al. 2003). Transgenic potato and cotton-carrying Cry03Aa offered resistance to Colorado beetle and boll weevil respectively (Wilson et al. 1992). Against the banana weevil, Bt gene with high toxic effects has not been identified so far.

Bakaze (2010) developed a diet to evaluate resistance in banana germplasm and in vitro efficacy of a *Bacillus thuringiensis* endotoxin Cry6A as well as *Carica papaya* cystatin (CpCYS) against *C. sordidus*. The artificial diet enabled banana weevil larvae to develop to adults in 48 days compared to 36 days on natural banana stem diet. The survival rate and the life cycle completion of the neonate were found more in media mixed with susceptible banana (Mbwazirume) corm powder than the resistant variety (Cavendish). The individual expression of these proteins was done in M15 cells by using pQE9/pQE30x9 expression system. The expressed proteins were purified and mixed at different concentration (1 ppm, 2 ppm) with diet. More than 65% of mortality was observed at 1 ppm concentration of Cry6A followed by CpCYS. A concentration of 2 ppm showed 83% mortality with CpCYS and 75% with Cry6A. Neonate mortality did not increase significantly when these proteins were mixed and used. Fifty percent mortality (LD50) of neonate larvae was observed at 0.24 ppm and 0.15 ppm for Cry6A and CpCYS, respectively. The outcome of this study showed that two genes together were more effective in combating *C. sordidus*.

Vegetative insecticidal proteins (VIPs) are another class of proteins which cause gut paralysis and lysis of the gut epithelium cells, thus

arresting gut function fully leading to the death of the insect (Duck and Evola 1997).

17.4.2.4 RNAi-Based Approaches

Ocimati et al. (2004) reported the 100 % mortality on banana weevil growth by using dsRNA synthesized from E2 ubiquitin gene which plays major role in protein catabolism of banana weevil. Significant growth retardation was observed with 50 and 100 ng/μl of dsRNA concentration in *in vitro* bioassay. Through this mechanism, essential genes can be silenced across the species, thus providing a molecular approach for great promise for the control of plant disease and pest (Whyard et al. 2009).

17.5 Genetic Transformation of Banana and Plantain for Nematode and Weevil Resistance

Transgenic banana of cultivar ‘Cavendish Williams’ expressing rice cystatin (OcIΔD86) was developed and tested in glasshouse for nematode resistance. These transgenic lines showed about 69–70 % resistance against *R. similis* (Atkinson et al. 2004b). Transgenic plantain of cultivar ‘Gonja manjaya’ was generated using a maize cystatin or the nAChR inhibiting peptide or both these traits stacked together (Roderick et al. 2012). Evaluation in the screen house for resistance against mixed population of the banana nematodes *R. similis*, *H. multincinctus* and *Meloidogyne* spp. identified several transgenic lines that provided 70–84 % resistance to *R. similis*. Numbers of *H. multincinctus* and *Meloidogyne* spp. were also suppressed, though the population on controls was too small to show statistical significance (Roderick et al. 2012). Further evaluation of the 12 lines with high levels of resistance in a confined field trial in Uganda identified a number of lines that matched resistance seen in the screen house. The best line, an nAChR inhibiting peptide line, reduced *R. similis* numbers by 99 % and provided a 186 % increase in yield compared to control plants (Tripathi

et al. 2015). This is the first field-based evidence of transgenic banana for resistance against nematodes.

Namuddu et al. (2013) developed transgenic banana cultivar ‘Sukali Ndiizi’ (ABB) using papaya cystatin (CpCYS-Mut89). This gene has been previously modified to improve its inhibitory potential against banana pests (Kiggundu et al. 2010). A total of 57 transgenic lines were generated. Putatively transgenic plants were validated by PCR, and gene integration was further confirmed by Southern blot hybridization. These transgenic lines are yet to be evaluated for resistance to weevils and nematodes.

17.6 Biosafety of Transgenic Nematode Resistance and Conclusion

Biosafety of transgenic approaches is an important consideration, particularly when intended for commercial release in a food crop. The safety of the engineered rice cystatin used in potato and banana field trials is well established. Cystatins are not toxins (Atkinson et al. 2004a), and plants expressing transgenic cystatins do not harm a range of nontarget organisms (Atkinson et al. 2009). Studies of impacts on free-living soil nematodes by transgenic anti-nematode plants have also been developed due to the high sensitivity of this group of organisms to changes in the soil microenvironment and them being the most likely group to be affected by anti-nematode defences (Ingham 2000). Nematode faunal analysis is used to quantify the shifts in free-living nematode genera across the different trophic groups present in the soil. Genera are split into those that respond rapidly to environmental change, which are used to calculate an enrichment index, and those that prefer undisturbed habitats, which are used to calculate a structural index. The ratio of enrichment to structural values indicates the state of the soil, and a shift in the ratio indicates disturbance (Ferris et al. 2001). When applied to a transgenic potato field trial with an nAChR-binding peptide and an engi-

neered rice cystatin, the transgenic lines did not significantly shift the structural or enrichment indices (Green et al. 2012).

Biosafety can be increased by reduction of transgenic defence expression outside of the roots through the use of tissue-specific promoters. Promoters have been used to deliver cystatins to the feeding sites of root-knot nematodes (Lilley et al. 2004). The tobacco cellulase promoter is expressed the syncytia of *H. schachtii* and has been used to develop an RNAi defence (Patel et al. 2008, 2010). The MDK4-20 promoter of *A. thaliana* has been used to target expression of an nAChR-binding peptide to root tips and also the root border cells that detach from the roots of many crops (Lilley et al. 2011a, b); when used to drive expression in potato, this promoter also provided greater levels of resistance compared to lines utilizing a constitutive promoter (Green et al. 2012). Such promoters can lower the burden of the transgene expression in transgenic plants, reduce nontarget organism exposure and increase food safety by preventing or reducing the presence of the transprotein in edible tissues.

RNAi-based defences lack the potential of allergenicity inherent in protein-based defences. It is, however, at risk of off-target effects both within the target organism and on nontarget organisms. Each double-stranded RNA (dsRNA) molecule needs to be carefully designed to reduce sequence identity between the target gene of the nematode and that any other sequence likely to be exposed to the dsRNA. Both of these considerations could be particularly important if small RNA molecules are shown to have prolonged environmental persistence (Auer and Frederick 2009).

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